Production of Enniatins by *Fusarium acuminatum* and *Fusarium compactum* in Liquid Culture: Isolation and Characterization of Three New Enniatins, B_2, B_3, and B_4†

Angelo Visconti,‡ Lisa A. Blais,§ John W. ApSimon,∥ Roy Greenhalgh,§ and J. David Miller*,§

Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada, Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, I-70126 Bari, Italy, and Ottawa-Carleton Chemistry Institute, Carleton University (DDT 1519), Ottawa, Ontario K1S 5B6, Canada

Three new enniatins, B_2, B_3, and B_4, were isolated from the hexane extracts of liquid cultures of *Fusarium acuminatum* MRC 3308, in addition to the four known enniatins, A, A_1, B, and B_1. The different enniatins were purified by reversed-phase semipreparative HPLC and could be detected directly in the extracts by FAB-MS or GC/MS. Enniatin B_2 was characterized as *N*-demethylenniatin B by its NMR, IR, and mass spectra. Enniatin B_3 was tentatively assigned the structure N,N^1-didemethylenniatin B on the basis of FAB-MS evidence. Enniatin B_4, an isomer of enniatin B_1, was shown by NMR to contain an *N*-methylleucine moiety. This is the first account of *N*-demethylenniatins (B_2 and B_3) being produced by fungi. Most of the tested strains of *F. acuminatum* (3 of 5 strains) and *Fusarium compactum* (8 of 10 strains) from different geographic origins produced one or more of the above enniatins.

INTRODUCTION

Depsipeptides constitute a large class of peptide-related compounds derived from hydroxy and amino acids joined by amide and ester linkages. Many members of this class are biologically active and include some antibiotics, alkaloids, and certain proteins (Shemyakin et al., 1969). Enniatins are cyclohexadepsipeptides and consist of three D-2-hydroxyisovaleric acid (Hylv) residues linked alternatively to L-amino acids or *N*-methyl-L-amino acid residues to give an 18-membered cyclic skeleton (Strongman et al., 1988). A number of enniatin analogues have been prepared by either chemical or microbiological synthesis. Types A and B enniatins, which have *N*-methylisoleucine (NMeIle) and/or *N*-methylvaline (NMeVal) moieties, are produced by fermentation of various strains of fusaria, including *Fusarium acuminatum* (Deol et al., 1978; Drysdale, 1984), *F. avenaceum* (Strongman et al., 1988), *F. compactum* (Greenhalgh et al., 1991), *F. lateritium* (Bishop and Isley, 1978), *F. oxysporum* and *F. scirpi* (Madry et al., 1983), *F. sambucinum* (Minasyan et al., 1978), and *F. tricinctum* (Burmeister et al., 1987). Enniatin C, with three *N*-methylleucine (NMeLeu) moieties, has only been obtained by chemical synthesis (Ovchinnikov et al., 1964).

The antibiotic, insecticidal, and phytotoxic activity of the enniatins has been studied extensively and appears to be related to their ionophoric properties (Shemyakin et al., 1969; Pressman, 1976). Enniatins are known to uncouple oxidative phosphorylation in isolated mitochondria, a reaction mediated by induction of an energy-dependent accumulation of potassium ions (Akimenko et al., 1981). They also affect water uptake by cells in tomato shoots, causing the symptoms of toxic wilt (Gaumann et al., 1960). Mixtures of enniatins produced by the entomopathogenic fungus *F. avenaceum* are known to be toxic at levels of 5 ppm to insect cells in culture (Strongman et al., 1988).

This paper describes the production and identification of several enniatins (Figure 1) from *F. acuminatum*, together with the HPLC and GC/MS procedures used for their isolation and analysis. Two new *N*-demethylenniatins (B_2 and B_3) and an *N*-methylleucine-containing enniatin (B_4) were isolated from *F. acuminatum* MRC 3308 and were characterized spectroscopically. Enniatin production from 5 strains of *F. acuminatum* and 10 strains of the related species *F. compactum* was determined.

MATERIALS AND METHODS

Instrumentation. *Infrared Spectroscopy (IR).* Spectra were obtained with either a Bomem Michaelson 100 FT-IR or a Perkin-Elmer 1600 Series FT-IR. All compounds were run as films on NaCl plates.

Mass Spectrometry (MS). Solid probe electron impact (EI) spectra were obtained on a Finnigan MAT 4500 GC/MS system with an INCOS data system: the probe was temperature programmed from 40 to 350 °C. GC/MS analyses were also performed with the same instrument equipped with a 30-m DB-5 capillary column. The column temperature was programmed from 120 to 300 °C over 15 min and then held at 300 °C for 15 min. Fast atom bombardment spectra (FAB) were recorded on a high-resolution Finnigan MAT 312 mass spectrometer equipped with an Ion Tech saddlefield atom gun. Diethanolamine (DEA) was used as the matrix for FAB spectra.

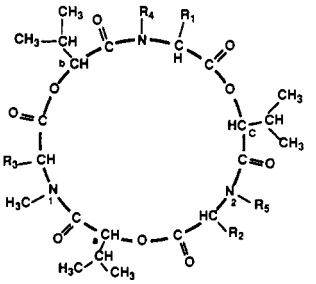
Nuclear Magnetic Resonance (NMR). ^1H and ^{13}C NMR spectra were determined on Bruker AM 250 and AM 500 NMR spectrometers equipped with ASPECT 3000 data systems. Chemical shifts were referenced to deuterochloroform at 7.24 and 77.0 ppm for ^1H and ^{13}C, respectively, and reported relative to tetramethylsilane (Me_4Si). ^{13}C chemical shift assignments were made by using a DEPT pulse sequence and by comparison with those of known enniatins. Confirmation of ^1H chemical shift assignments was made by the use of $^1H/^1H$ homonuclear correlation spectra (COSY) and by specific decoupling.

High-Pressure Liquid Chromatography (HPLC). A Varian Vista 5500 series chromatograph equipped with a Varian 2550 UV detector (245 nm) was used. For preparative HPLC, a 10-μm Whatman Magnum 9 Partisil ODS-2 RP column (9.4 mm i.d. × 25 cm) was employed with solvent systems of 85% acetonitrile/water for the initial separation and 70% acetonitrile/water for further purification of the different fractions at a flow rate of 4 mL min^{-1}.

Chemicals. A standard of enniatin B_1 was kindly provided by R. D. Plattner, U.S. Department of Agriculture, Peoria, IL.

* Author to whom correspondence should be addressed.
† PRC Contribution No. 1380.
‡ Istituto Tossine e Micotossine de Parassiti Vegetali.
§ Agriculture Canada.
∥ Carleton University.

0021-8561/92/1440-1076$03.00/0



ENNIATIN	R_1	R_2	R_3	R_4	R_5
A	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	CH_3	CH_3
A_1	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)_2$	CH_3	CH_3
B_1	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$	CH_3	CH_3
B_4	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH_2-CH(CH_3)_2$	CH_3	CH_3
B	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)_2$	CH_3	CH_3
B_2	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)_2$	H	CH_3
B_3	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)_2$	H	H

Figure 1. Structure of the enniatins produced by *F. acuminatum* MRC 3308.

Enniatins A, A_1, and B have been isolated previously in our laboratory from *F. avenaceum* DAOM 196490.

Fermentation. The following strains of *F. acuminatum* were studied: MRC 3308, MRC 3309, MRC 3397, and MRC 3826 from South Africa (Lamprecht et al., 1986; Rabie et al., 1986); DAOM 180361 isolated from soil in Manitoba, Canada. In addition, 10 strains of *F. compactum* were examined. ITEM 484 and ITEM 488 were isolated in Italy (Visconti et al., 1989). Strains R8285, R8286, R8287, R8288, and R8293 were isolated in Texas, from a Sandhill crane intoxication. Strain R6784 was isolated from river sediment in Japan, R6910 from an air sample from Queensland, Australia, and R7515 from soil from Texas. The strains with the prefix "R" were classified and provided by P. Nelson, *Fusarium* Research Center, Pennsylvania State University.

Seed cultures were prepared by macerating slants of the fungi in sterile distilled water (25 mL). Aliquots (2.5 mL) of the resulting suspension were used to inoculate Erlenmeyer flasks (250 mL) each containing CZ-MET medium (50 mL) autoclaved at 121 °C for 12 min. The cultures were put on a rotary shaker at 220 rpm at 28 °C for 48 h. The resulting mycelia were macerated and inoculated (5% v/v) in Erlenmeyer flasks (250 mL) containing 50 mL of CZ-MET medium (typically three flasks). The cultures were put on a rotary shaker at 220 rpm at 28 °C for 7 days and then filtered and the mycelia dried at 40 °C under vacuum. *F. acuminatum* MRC 3308 produced the greatest quantity of enniatins. This culture was grown in 40 flasks to provide more material for examination. The details of the fermentation are the same as above except that an alternative production medium was used. It consisted of 50 g of maltose, 8 g of peptone, 5 g of yeast extract, 0.75 g of KH_2PO_4, 0.50 g of $MgSO_4 \cdot 7H_2O$, and 0.007 g of $CaCl_2 \cdot H_2O$ per liter of ultrapure water.

Extraction and Purification. Cultures grown for screening purposes were filtered through Whatman No. 1 filter paper to remove the mycelia. The broth (100 mL) from each strain was transferred to a Clin Elut column and extracted with ethyl acetate (5 × 25 mL). Solvent was removed in vacuo, and the residue was dissolved in methanol. Extracts were screened for production of enniatins by GC/MS and FAB-MS.

The large-scale fermentation of *F. acuminatum* MRC 3308 was filtered to remove the mycelia, and the resulting broth (2 L) was extracted first with hexane (3 × 2 L) and then ethyl acetate (3 × 2 L). Removal of the solvents yielded 523 and 338 mg, respectively, for the two extracts. The mycelium was blended with dichloromethane, and the residue from the extract was partitioned between hexane and methanol/water (50:50) (Blais, 1991).

The hexane extract of the culture broth was dissolved in acetonitrile (5 mL) and chromatographed by HPLC using acetonitrile/water (85:15) as the mobile phase. Five fractions were collected and analyzed by FAB-MS and ^1H NMR: Fraction 1 (4 mg) contained a mixture of compounds, including enniatins B_3 and B_2. Fraction 2 (33 mg) was mainly enniatin B, but the ^1H NMR spectrum indicated the presence of a second compound with similar structure. Fraction 3 (9 mg) was shown to contain some enniatin B_1, fraction 4 (4 mg) enniatin A_1, and fraction 5 (2 mg) enniatin A, which were identified by comparison with standards. Both fractions 1 and 2 were purified further by HPLC using a solvent system of 70:30 acetonitrile/water. The major compound of fraction 1 (enniatin B_2) was isolated as a pale yellow oil (2 mg): IR 3400 (weak), 2966, 1742, 1659 cm^{-1}; solid probe EI-MS *m/z* 42 (44), 55 (29), 69 (48), 86 (base), 98 (18), 130 (12), 141 (18), 154 (18), 168 (30), 182 (23), 196 (56), 214 (17), 496 (11), 526 (12), 542 (12), 625 (M^+); ^1H and ^{13}C NMR (see Tables I and II).

In the chromatogram of fraction 2, the peak for enniatin B showed a shoulder. This mixture was further resolved to yield pure enniatin B (15 mg) and a similar amount (14.0 mg) of a second compound (enniatin B_4) as a pale yellow oil: $[\alpha]_D$ −88.9° (*c* 0.09, $CHCl_3$, 25 °C); IR, 2966, 1746, 1660 cm^{-1}; solid probe EI-MS *m/z* 42 (56), 55 (24), 69 (36), 86 (base), 100 (46), 112 (9), 126 (11), 141 (20), 155 (20), 169 (34), 183 (34), 196 (80), 210 (29), 282 (12), 296 (16), 396 (14), 410 (14), 423 (17), 441 (12), 457 (5), 470 (5), 524 (13), 552 (18), 571 (18), 653 (M^+); ^1H and ^{13}C NMR (see Tables I and II).

RESULTS AND DISCUSSION

F. acuminatum MRC 3308 gave both the highest yield and the most diverse mixture of enniatins. In addition to the known enniatins A, A_1, B, and B_1, the FAB-MS spectrum of the hexane extract of the culture media also showed the presence of two new enniatins, designated B_2 (MW 625) and B_3 (MW 611). The molecular ions (M^+ + H) for enniatins B_2, B, and B_1 were observed at *m/z* 626, 640, and 654, respectively. The potassium adduct ions of enniatins B and B_1 were also observed at *m/z* 678 and 692, respectively. Adducts of the various enniatins with diethanolamine (DEA, MW 105.5) resulted in ions (M^+ + DEA) at *m/z* 731 (B_2), 745 (B), 759 (B_1), 773 (A_1), and 787 (A). The ions at *m/z* 717 (M^+ + DEA), 650 (M^+ + K), 634 (M^+ + Na), and 612 (M^+ + H) suggested the presence of an enniatin-like compound with molecular weight of 611 (enniatin B_3). Enniatins B, B_1, A_1, and A were also found in the ethyl acetate extract of the medium filtrate as well as in the hexane extract of the mycelium. GC/MS analysis gave satisfactory results in terms of resolution and the immediate identification of enniatins by their mass spectra. Enniatins were eluted in the order B_2, B_3, B, B_1, A_1, and A. The use of high column temperatures for long periods and column overloading by the major enniatins present in fungal cultures resulted in poor resolution and shorter column life.

The different enniatins were separated by reversed phase HPLC.

Solid probe EI-MS of enniatin B_2 showed a molecular ion of *m/z* 625 corresponding to the (M^+ + H) ion at *m/z* 626.5 in the FAB-MS. The fragmentation pattern was similar to those of other enniatins. The difference of 14 mass units in the molecular weight as compared to enniatin B suggests that either a terminal CH_3 from an isopropyl side chain or an *N*-methyl group is lost.

The IR spectrum of enniatin B_2 showed a small, broad band at about 3400 cm^{-1}, which could be attributed to the NH stretching frequency of a secondary amide. This would suggest a structure for enniatin B_2 similar to that of enniatin B but with the loss of a methyl group from one of the nitrogen atoms.

The assignment of resonances in the ^1H NMR spectrum of enniatin B_2 is reported in Table I. A doublet at 6.80 ppm which showed allylic coupling to a doublet of doublets at 4.60 ppm was assigned to the NH group, in agreement with the ^1H data for tri-*N*-demethylenniatin B (Ovchinnikov et al., 1974). The resonance at 4.60 ppm (αCH, Val), in turn, showed coupling to a multiplet (βCH) at 2.22 ppm. The γCH_3 protons were assigned to the

Table I. 500-MHz ^{1}H NMR Chemical Shift Assignments for Enniatins B_2, B_4, B_1, and B

	proton	B_2	B_4	B_1[a]	B[b]
NMeVal	αH	4.82 ($J_{\alpha,\beta}$ = 10.6)	4.89 ($J_{\alpha,\beta}$ = 10.2)	4.42	4.42
		3.48 ($J_{\alpha,\beta}$ = 8.7)	4.44 ($J_{\alpha,\beta}$ = 10.0)	4.46	
	βH	2.38	2.23	2.27	2.27
		2.47	2.16		
	γH$_3$	0.98	1.02 ($J_{\gamma,\beta}$ = 6.2)	1.02	1.03
		0.95	0.85 ($J_{\gamma,\beta}$ = 6.8)	0.87	0.86
		1.07 ($J_{\gamma,\beta}$ = 6.6)			
		0.95			
	N—Me	3.14	3.07	3.09	3.09
		2.88	3.10		
NHVal	αH	4.60 ($J_{\alpha,\beta}$ = 3.9) ($J_{\alpha,NH}$ = 7.9)			
	βH	2.22			
	γH$_3$	0.84 ($J_{\gamma,\beta}$ = 6.7)			
		0.92–0.99			
	NH	6.80 ($J_{NH,\alpha}$ = 8.0)			
NMeLeu	αH		4.66	[4.65][c]	
	βH$_2$		1.73 ($J_{A,B}$ = 13.5)	[2.04]	
	[βH]		1.81 ($J_{\beta,\gamma}$ = 5.1) ($J_{\beta,\alpha}$ = 10.1)		
	γH[γH$_2$]		1.55	[1.04, 1.42]	
	[γH$_3$]			[0.98]	
	δH$_3$		0.91 ($J_{\delta,\gamma}$ = 6.6)	[0.82]	
			0.92 ($J_{\delta,\gamma}$ = 6.6)		
	N—Me		3.04		
HyIv	αH	5.46(A) ($J_{\alpha,\beta}$ = 9.9)	4.98 ($J_{\alpha,\beta}$ = 9.0)	5.11	5.11
		4.84(B) ($J_{\alpha,\beta}$ = 8.8)	5.09 ($J_{\alpha,\beta}$ = 8.5)		
		5.04(C) ($J_{\alpha,\beta}$ = 9.8)	5.18(Leu) ($J_{\alpha,\beta}$ = 8.4)		
	βH	2.35(A)	2.24	2.24	2.27
		2.01(B)	2.22		
		2.16(C)	2.27(Leu)		
	γH$_3$	0.95, 0.89(A)	0.93–0.97	0.92–0.98	0.93
		0.95, 1.00(B)		0.98	0.96
		0.83, 1.01(C)			

[a] Blais et al. (1992). [b] Blais (1991). [c] Values in square brackets are for NMeIle.

Table II. 125-MHz ^{13}C NMR Chemical Shift Assignments for Enniatins B_2, B_4, B_1, and B

	carbon	B_2	B_4	B_1[a]	B_b
NMeVal	αC	67.8	61.3	63.5	63.2
		56.7	63.2	63.4	
	βC	30.7	27.5	27.9	27.9
		27.9	27.8		
	γC	17.0, 25.6	19.9, 20.3	19.4, 20.5	19.3
					20.4
	N—Me	37.3	33.0	33.4	33.2
		31.0	33.8		
	C=O(amide)	169.6, 168.9	169.0, 169.3	169.3	169.3
	C=O(ester)	170.6, 170.4	170.3, 170.4	170.4	170.3
NHVal	αC	60.9			
	βC	28.4			
	γC	17.0–25.6			
	C=O(amide)	167.7			
	C=O(ester)	170.4			
NMeLeu	αC		57.2	[61.5][c]	
	βC		37.9[d]	[33.8]	
	γC		25.2	[25.3,[d] 16.1]	
	δC		21.5, 23.3	[10.9]	
	N—Me		31.6	[32.7]	
	C=O(amide)		169.6	[169.3]	
	C=O(ester)		170.7	[170.4]	
HyIv	αC	78.6, 74.2, 75.4	74.9, 75.2, 75.6	77.2, 75.0, 75.6	75.7
	βC	31.4, 29.6, 29.9	29.6, 29.8, 30.2	29.7, 29.8, 30.3	29.9
	γC	17.0–25.6	18.3–18.9	18.2–18.9	18.5, 18.6

[a] Blais et al. (1992). [b] Blais (1991). [c] Values in square brackets are for NMeIle. [d] CH_2.

resonances at 0.84 and 0.92–0.99 ppm on the basis of a COSY spectrum (Blais, 1991). The adjacent hydroxy acid [HyIv(B)] was assigned the resonances at 4.84 (αH), 2.01 (βH), and 0.95 and 1.00 ppm (γCH$_3$). In the ^{1}H spectrum of enniatin B, the αCH group of NMeVal appears as a doublet at about 4.42 ppm. However, the spectrum of enniatin B_2 has no doublets in this region. From the COSY spectrum, the αCH of one of the NMeVal resonances [NMeVal(1)] was assigned to the doublet at 4.82 ppm. This doublet showed coupling to a multiplet at 2.38 ppm (βH), which in turn coupled to two doublets at 0.95 and 0.98 ppm. The αCH of the other NMeVal moiety

[NMeVal(2)] was assigned to a doublet at 3.48 ppm, on the basis of specific decoupling experiments. When the multiplet at 2.47 ppm was irradiated, a decrease was observed in the intensity of this doublet and the doublet at 1.07 ppm. The latter was assigned to one of the γCH_3 groups. The other methyl group was assigned to a resonance at 0.95 ppm from the COSY spectrum. The two methyl singlets at 2.88 and 3.14 ppm were assigned to N–CH_3 of NMeVal(2) and NMeVal(1), respectively. The resonances at 5.04, 2.16, 0.83, and 1.01 ppm were assigned to the hydroxy acid [HyIv(C)] adjacent to NMeVal(2). Similarly, the other HyIv(A) moiety was assigned the resonances at 5.46, 2.35, 0.95, and 0.89 ppm.

The loss of the methyl group attached to one nitrogen in enniatin B_2 as compared to enniatin B, and the possible hydrogen bonding between the NH group and the skeleton, leads to a substantial change of ring conformation demonstrated by a nonequivalence of the NMeVal and HyIv moieties in enniatin B_2. This explains the appearance of discrete resonances for the N–CH_3 and the αCH of NMeVal and the HyIv in enniatin B_2 as opposed to a single signal in enniatin B for these protons (singlet at 3.09 ppm for N–CH_3; doublet at 4.42 ppm for αCH of NMeVal; doublet at 5.11 ppm for αCH of HyIv). The wide range in chemical shift for the resonances of the α-proton of NMeVal (4.82–3.48 ppm) is not surprising as chemical shift differences of up to 2 ppm have been observed for equivalent α-protons of *N*-methylamino acids in cyclic peptides. The ^1H NMR spectrum of the cyclic heptapeptide rhizonin, a mycotoxin produced by *Rhizopus microsporus*, provides a parallel example (Potgieter, 1986).

The 125-MHz ^{13}C NMR spectrum of enniatin B_2 shows 32 resonances, consistent with the assigned molecular weight of 625. Assignments for these resonances are given in Table II.

Attempts to isolate pure enniatin B_3 were unsuccessful due to the small amount of material present in the crude extract. However, it was possible to detect the compound in the enniatin B_2 fraction. A sample enriched in enniatin B_3 was studied by GC/MS and FAB-MS (Figure 2). The EI-MS of enniatin B_3 shows all of the diagnostic fragments reported in Figure 3 but no molecular ion. In addition, ions at *m/z* 382, generated by the loss of HyIv and NMeVal moieties, 86, 196 and 296, common to all type B enniatins, 568 ($M^+ - 43$), and 528 ($M^+ - 83$) were observed. The MW of enniatin B_3 was shown to be 611 by the presence of M + H, M + Na, M + K, and M + DEA adducts in the FAB-MS spectrum (Figure 2B). It indicates the loss of 28 amu when compared to enniatin B, suggesting loss of two methyl groups on nitrogen atoms for B (or one methyl group for B_2), resulting in *N*,*N*^{1}-di-*N*-demethylenniatin B.

The other new enniatin, B_4, which was isolated by HPLC from the fraction containing enniatin B, gave a molecular ion (M^+) at *m/z* 653 by solid probe EI-MS, in accord with an ($M^+ + H$) ion at *m/z* 654.5 by FAB-MS. Although the molecular weight is identical to that of enniatin B_1, their HPLC retention times differ by 1.5 min using acetonitrile/water (70:30) as the solvent system. Since enniatins B_1 and B_4 appear to be isomeric, it is postulated that the NMeIle moiety of B_1 could have been replaced by an NMeLeu moiety.

The ^1H NMR spectrum (250 MHz) of enniatin B_4 integrates for 59 protons in accord with the proposed structure. The resonance at 4.66 ppm was assigned to the αCH of the NMeLeu moiety and showed allylic coupling to the βCH_2 multiplet at 1.73 and 1.81 ppm. The latter gave crosspeaks to another multiplet at 1.55 ppm (γCH)

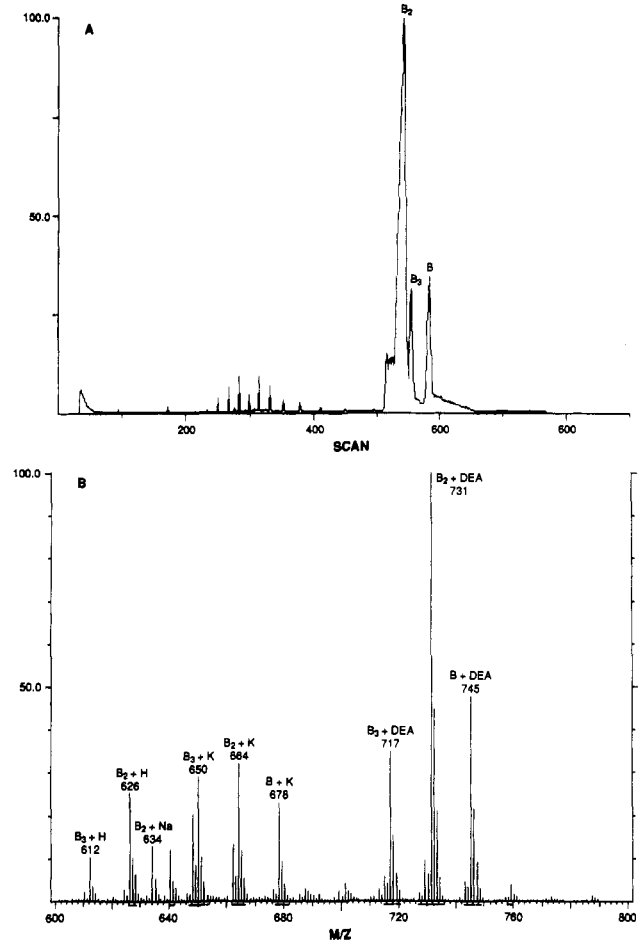
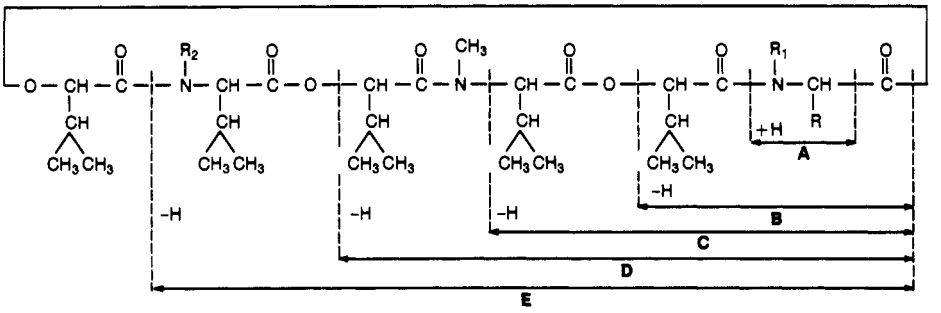


Figure 2. GC/MS reconstructed ion chromatogram (A) and FAB-MS spectrum (B) of an HPLC fraction of culture extract of *F. acuminatum* MRC 3308, containing enniatins B_2, B_3, and B. Ions of the respective enniatin adducts with H, Na, K, and DEA (diethanolamine) are shown. Experimental conditions are described in the text.

in the COSY spectrum. Finally, coupling between the γCH and the two doublets (3 H each) at 0.91 and 0.92 ppm confirmed them as the δCH_3 groups of NMeLeu.

Assignment of the MeVal resonances of enniatin B_4 was made from the COSY spectrum and by comparison with ^1H NMR data for enniatins B and B_1 (Table I). The resonances at 4.44 (αCH), 2.23 (βCH), 0.84 and 1.02 (C–γCH_3), and 3.10 ppm (N–CH_3), attributed to one NMeVal moiety, were similar to the corresponding ones for enniatins B and B_1. In contrast, the other NMeVal moiety showed a downfield shift for the αCH resonance to 4.84 ppm and upfield shifts of the βCH and N–CH_3 resonances to 2.16 and 3.07 ppm, respectively. The chemical shift differences may be explained by the loss of symmetry when one of the isopropyl side chains in the molecule is changed to an isobutyl group.

The downfield doublets at 5.18, 5.09, and 4.98 ppm were assigned to the αCH protons of the HyIv residues adjacent to NMeLeu and two NMeVal residues, respectively, on the basis of COSY data and in comparison to enniatin A_2. The latter, which is the isomer of enniatin A having one NMeLeu instead of NMeIle, shows resonances for the αCH protons of the HyIv groups at 5.18 (next to the NMeLeu), 5.05, and 4.92 ppm (Blais et al., 1992). Resonances at 2.24 and 2.22 ppm were assigned to the βCH of HyIv next to NMeVal, and the resonance at 2.27 ppm was assigned to the HyIv moiety next to NMeLeu. The γCH_3 groups of the three HyIv residues appear as overlapping doublets in the region 0.93–0.97 ppm. Finally, as for enniatin A_2, the



Enniatin	R_1	R_2	R	A	B	C	D	E	M^+
B	CH_3	CH_3	iPr	86*	196*	296*	409	538	639
B_2	H	CH_3	iPr	72	182	282	395	524	625
B_3	H	H	iPr	72	182	282	395	510	–
B_1	CH_3	CH_3	sBu[a]	100	210	310	423	552	653
B_4	CH_3	CH_3	iBu[b]	100	210	310	423	552	653

*also present in enniatins B_1, B_2, B_3 and B_4 spectra
(a) secbutyl from NMeIle
(b) isobutyl from NMeLeu

Figure 3. Mass spectral fragmentation patterns for enniatins B, B_1, B_2, B_3, and B_4.

singlet (3 H) at 3.04 ppm was assigned as the N-CH_3 group of NMeLeu.

The 62.5-MHz ^{13}C NMR spectrum of enniatin B_4 shows only 32 resonances, in contrast to the 34 carbons in the molecule. A DEPT spectrum (135°) of the compound showed the presence of only one CH_2 group, at 37.9 ppm, in agreement with the presence of one NMeLeu but no NMeIle moiety, in which the γCH_2 would occur at 25.3 ppm. A DEPT (90°) experiment indicated the presence of only 10 CH carbons; the αCH resonances for HyIv-NMeLeu and NMeLeu at 75.6 and 57.2 ppm, which were present in the normal ^{13}C spectrum, were not detected. The resonances at 57.2 (αCH), 37.9 (βCH_2), 25.2 (γCH), 23.3 and 21.5 (δCH_3), and 33.8 ppm (N-CH_3) were assigned to the NMeLeu residue on the basis of ^{13}C NMR data for leucine and by comparison to ^{13}C NMR and HETCOR data for enniatin A_2 (Blais et al., 1992). Comparison with the spectra of enniatins B and B_1 enabled assignment of the resonances at 63.2 and 61.3 ppm to the αCH protons of the NMeVal moieties and resonances at 33.8 and 33.0 ppm to the relevant *N*-methyl groups. The remainder of the ^{13}C NMR assignments for enniatin B_4 are given in Table II.

Whereas FAB-MS is an excellent technique for monitoring fungal extracts for the presence of enniatins, the fragmentation patterns of EI spectra are more definitive for structural characterization. Enniatins B, B_1, B_2, B_3, and B_4 exhibit a cluster of diagnostic ions at *m/z* 86, 196, and 296 associated with the fragments A, B, and C, respectively as shown for enniatin B (R_1 = Me; R = iPr) (Figure 3). The corresponding ions for enniatins A, A_1, and A_2 (R_1 = Me; R = iBu) are observed at *m/z* 100, 210, and 310. In the mass spectrum of enniatin B_2 (R_1 = H; R = iPr), the intensity of the ions at *m/z* 72, 182, and 282 is less than that of the ions at *m/z* 86, 196, and 296 which are derived from the two NMeVal moieties (R_1 = Me; R = iPr). Similarly, for enniatins B_1 and B_4 (R_1 = Me; R = iBu) the intensities of fragments at *m/z* 100, 210, and 310 are less than those of the corresponding fragments derived from the two NMeVal moieties (*m/z* 86, 196, and 296).

The initial fragmentation of the enniatins is accompanied by the loss of the HyIv fragment containing one more hydrogen atom [Figure 3, 101 amu $(CH_3)_2CHCH_2COO^-$]. The corresponding fragments (E, Figure 3) for enniatins B_4/B_1, B, B_2, and B_3 are 552, 538, 524, and 510 amu, respectively.

Table III. Screening of *F. acuminatum* and *F. compactum* Isolates for Enniatin Production

isolate	enniatins
F. acuminatum	
MRC 3308	B, B_1, B_2, B_3, B_4, A, A_1, A_2
MRC 3309	B, B_1, B_2, A, A_1
MRC 3397	nd[a]
MRC 3826	nd
DAOM 180361	B, B_1, B_2, A, A_1
F. compactum	
ITEM 484	nd
ITEM 488	nd
R6784	B
R6910	B, A, A_1
R7515	B, B_1, B_2, A, A_1
R8285	B, B_1, A, A_1
R8286	B, B_1, A, A_1
R8287[b]	B
R8288	B, A, A_1
R8293[b]	B, B_1

[a] nd, not detected. [b] See Greenhalgh et al. (1991).

The further loss of NMeVal (129 amu) for enniatins B, B_1, B_2, and B_4, or Val (115 amu) for enniatin B_3, generates fragments at *m/z* 423, 409, 395, and 381 for enniatins B_4/B_1, B, B_2, and B_3, respectively (D, Figure 3). The loss of side-chain fragments (C_4H_9 or C_3H_7) from the parent ion M^+ results in ions at *m/z* 596 ($M^+ - 57$), 596 ($M^+ - 43$), 582 ($M^+ - 43$), and 568 ($M^+ - 43$) for enniatins B_4/B_1, B, B_2, and B_3, respectively. Another ion which occurs at high mass is that of $M^+ - 83$, resulting from the loss of the fragment $(CH_3)_2CHC{=}C{=}O$ from the HyIv moiety, although these ions are not shown in Figure 3 yet are diagnostic for enniatins.

F. acuminatum occurs worldwide as a soil saprophyte and as a secondary pathogen associated with plant diseases. In addition to producing enniatins, this species is known to produce trichothecenes, such as T-2 toxin and DAS (Marasas et al., 1984), as well as acuminatopyrone and chlamydosporol (Grove and Hitchcock, 1991). The data concerning the production of enniatins by *F. acuminatum* and *F. compactum* are shown in Table III. Three strains of *F. acuminatum* from South Africa and Canada produced the enniatins B, B_1, A, and A_1. Two strains,

MRC 3397 and MRC 3826, produced the trichothecene T-2 (Rabie et al., 1986) but did not produce enniatins under the conditions tested. *F. acuminatum* MRC 3308 produced a number of new enniatins including B_4 and B_2 and B_3 as minor components, i.e., less than 10% of the total enniatins. The production of enniatins by *F. acuminatum* has already been reported by Deol et al. (1978) as a mixture of enniatins A (3%), A_1 (17%), B_1 (41%), and B (39%). Our results confirm the preponderance of the type B enniatins over type A enniatins for this species. This is reversed in the case of *F. avenaceum*, however, where the type A enniatins predominate (Blais et al., 1992; Strongman et al., 1990).

Enniatin B and B_1 production by *F. compactum* was first reported by Greenhalgh et al. (1991). This involved strains isolated from a Sandhill crane toxicosis in Texas. Three additional strains isolated from samples of the contaminated feed (R8285, 8286, 8288) also produced enniatins B and B_1 plus A and A_1 (Table III). Strain R7515, isolated from soil from Texas, produced the latter four enniatins plus the new enniatin B_2. Strain R6910 isolated in Australia produced enniatins B, A, and A_1, while only enniatin B was detected from the Japanese strain R6784. The two strains from Italy, ITEM 484 and 488, did not produce enniatins under the conditions tested. The latter strains have been shown to produce a number of trichothecenes, including acuminatin (Visconti et al., 1989). These data include examples of strains of *F. acuminatum* and *F. compactum* that produce trichothecenes but not enniatins.

The production of enniatins containing an NMeLeu moiety by *Fusarium* has only been shown recently by our group with the isolation and characterization of enniatin A_2, where the three HyIv units are esterified with two NMeIle and one NMeLeu (Blais et al., 1992). Enniatin B_4 represents an additional compound of this type produced by *Fusarium*. This is the first report of *N*-demethylenniatins in nature. The conformational changes of the enniatin skeleton induced by the loss of methyl groups attached to nitrogen would be expected to lead to substantial changes in their biological activities.

Most of the available data on the biological activity of enniatins are referred to mixtures of two or more enniatins or to pure enniatins A and B. In particular, the insecticidal activity of the mixture of type A enniatins has been shown by Strongman et al. (1990) against *Choristoneura fumiferana* Clem. (spruce budworm) and by Grove and Pople (1980) against *Calliophora erythrocephala* (blowfly) and *Aedes aegypti* (mosquito) larvae. The mixtures B/B_1 and A/B have been shown to be phytotoxic by Burmeister and Plattner (1987) and Gaumann et al. (1960), respectively. Enniatins A and B have been used to test phytotoxicity and antimicrobial activity by Gaumann et al. (1960) and Tirunarayanan and Sirsi (1957).

In this study we have isolated by semipreparative HPLC six enniatins, namely A, A_1, B, B_1, B_2, and B_4, as pure compounds from a fungal culture extract (enniatin B_3 could only be obtained in a mixture together with B and B_2). These enniatins are representative of possible structural variations. Attention is drawn to the new enniatins B_2, B_3, and B_4 which exhibit novel structural differences, such as the N-demethylation or the presence of an NMeLeu moiety, compared to the well-studied enniatins A and B. The availability of various enniatins as pure compounds will facilitate further study of possible synergistic effects as well as further work on the structure/activity correlation for this class of compounds.

ACKNOWLEDGMENT

We thank G. Montgomery for technical assistance, P. Lafontaine for mass spectral data, and B. A. Blackwell for help with analysis of the NMR data. This work was supported in part by the Commission of European Communities fund for EC-Canada cooperation and by an NSERC postgraduate scholarship to L.B. We thank G. A. Neish, Agriculture Canada, for identification of *F. acuminatum* strain DAOM 180361.

LITERATURE CITED

Akimenko, V. K.; Andreeva, N. A.; Kulaev, I. S.; Lozinov, A. B.; Chermenskii, D. N. Relation between enniatin synthesis, ATP pool, and cyanide-resistant respiration in *Fusarium sambucinum*. *Mikrobiologiya* **1981**, *50*, 205–210.

Bishop, G. C.; Isley, A. H. Production of enniatin as a criterion for confirming the identity of *Fusarium lateritium* isolates. *Aust. J. Biol. Sci.* **1978**, *31*, 93–96.

Blais, L. A. M.Sc. Thesis, Ottawa-Carleton Chemical Institute, Carleton University, Ottawa, ON, Canada, 1991.

Blais, L. A.; ApSimon, J. W.; Blackwell, B. A.; Greenhalgh, R.; Miller, J. D. Isolation and characterization of minor enniatins from *Fusarium avenaceum* Fa120. *Can. J. Chem.* **1992**, in press.

Burmeister, H. R.; Plattner, R. D. Enniatin production by *Fusarium tricinctum* and its effect on germinating wheat seeds. *Phytopathology* **1987**, *77*, 1483–1487.

Deol, B. S.; Ridley, D. D.; Singh, P. Isolation of cyclodepsipeptides from plant pathogenic fungi. *Aust. J. Chem.* **1978**, *31*, 1397–1399.

Drysdale, R. B. The production and significance in phytopathology of toxins produced by species of *Fusarium*. In *The Applied Mycology of Fungi*; Moss, M. O., Smith, J. E., Eds.; Cambridge University Press: Cambridge, 1984; pp 95–106.

Gaumann, E.; Naef-Roth, St.; Kern, H. Phytotoxic activity of the enniatins. *Phytopathol. Z.* **1960**, *40*, 45–51.

Greenhalgh, R.; Miller, J. D.; Visconti, A. Toxigenic potential of *Fusarium compactum* R8287 and R8293. *J. Agric. Food Chem.* **1991**, *39*, 809–812.

Grove, J. F.; Hitchcock, P. B. Metabolic products of *Fusarium acuminatum*: acuminatopyrone and chlamydosporol. *J. Chem. Soc., Perkin Trans. 1* **1991**, 997–999.

Grove, J. F.; Pople, M. The insecticidal activity of beauvericin and the enniatin complex. *Mycopathologia* **1980**, *70*, 103–105.

Lamprecht, S. C.; Marasas, W. H. O.; Thiel, P. G.; Schneider, D. J.; Knox-Davies, F. S. Incidence and toxicity of seedborne *Fusarium* species from annual *Medicago* species in South Africa. *Phytopathology* **1986**, *76*, 1040–1042.

Madry, N.; Zocher, R.; Kleinkauf, H. Enniatin production by *Fusarium oxysporum* in chemically defined media. *Eur. J. Appl. Microbiol. Biotechnol.* **1983**, *17*, 75–79.

Marasas, W. F. O.; Nelson, P. E.; Toussoun, T. A. *Toxigenic Fusarium species: Identity and Mycotoxicology*; Pennsylvania State University Press: University Park, PA, 1984.

Minasyan. A. E.; Chermenskii, D. N.; Ellanskaya, I. A. Synthesis of Enniatin B by *Fusarium sambucinum*. *Mikrobiologiya* **1978**, *47*, 67–71.

Ovchinnikov, Yu. A.; Ivanov, V. T.; Evstratov, A. V.; Mikhaleva, I. I.; Bystrov, V. F.; Portnova, S. L.; Balashova, T. A.; Meshcheryakova, E. N.; Tulchinsky, V. M. The enniatin ionophores. Conformation and ion binding properties. *J. Pept. Protein Res.* **1974**, *6*, 465–498.

Plattner, P. A.; Nager, U. Analysis and characterization of enniatins. The separation of N-methyl amino acids by paper chromatography. *Helv. Chim. Acta* **1948**, *31*, 2203–2209.

Potgieter, M. Conformational analysis of the cyclic peptide rhizonin. In *Mycotoxins and Phycotoxins*; Steyn, P. S., Vleggaar, R., Eds.; Elsevier Science Publishers: Amsterdam, 1986; pp 205–214.

Pressman, B. C. Biological applications of ionophores. *Annu. Rev. Biochem.* **1976**, *45*, 501–530.

Rabie, C. J.; Sydenham, E. W.; Thiel, P. G.; Luebben, A.; Marasas, W. F. O. T-2 toxin production by *Fusarium acuminatum* isolated from oats and barley. *Appl. Environ. Microbiol.* **1986**, *52*, 594–595.

Shemyakin, M. M.; Ovchinnikov, Y. A.; Ivanov, V. T.; Antonov, V. K.; Vinogradova, E. I.; Shkrob, A. M.; Malenkov, G. G.; Evstratov, A.; Laine, I. A.; Melnik, E. I.; Ryabova, I. D. Cyclodepsipeptides as chemical tools for studying ionic transport through membranes. *J. Membr. Biol.* **1969**, *1*, 402–430.

Strongman, D. B.; Strunz, G. M.; Giguere, P.; Yu. C.-M.; Calhoun, L. Enniatins from *Fusarium avenaceum* isolated from balsam fir foliage and their toxicity to spruce budworm larvae, *Choristoneurea fumiferana* (Clem.). *J. Chem. Ecol.* **1988**, *14*, 753–764.

Tirunarayanan, M. O.; Sirsi, M. Antibiotics from the genus Fusarium. Enniatin B. I. Culture studies and antimicrobial activity. *J. Indian Inst. Sci.* **1957**, *39*, 185–194.

Visconti, A.; Mirocha, C. J.; Logrieco, A.; Bottalico, A.; Solfrizzo, M. Mycotoxins produced by *Fusarium acuminatum*. Isolation and characterization of Acuminatin: a new trichothecene. *J. Agric. Food Chem.* **1989**, *37*, 1348–1351.

Received for review November 4, 1991. Accepted March 2, 1992.

Registry No. Enniatin A, 2503-13-1; enniatin A_1, 4530-21-6; enniatin B, 917-13-5; enniatin B_1, 19914-20-6; enniatin B_2, 632-91-7; enniatin B_3, 864-99-3; enniatin B_4, 19893-21-1.